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14. ABSTRACT

We have accomplished the generation of mice with an altered Rheb1 gene expression. In addition, we have also successfully generated an antibody which is highly specific for mouse Rheb2. Investigation into the function of Rheb GTPase and its significance in Tuberous Sclerosis is being studied.

15. SUBJECT TERMS
Rheb GTPase, Tuberous Sclerosis, Gene Knockout, Antibody

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Introduction

This report covers the period December 15, 2004-December 14, 2005. We have made progress in generating a mouse model to study the function of Rheb. Mice with decreased Rheb1 expression have been generated and are being characterized. In addition, we have initiated the project to produce an antibody specific for Rheb2. Details of our progress are explained in the body of this report.

Summary of progress

Our overall aim is to study a unique member of the Ras superfamily G-protein called Rheb which is a critical component of the insulin/TSC/mTOR pathway (Aspuria and Tamanoi, 2004; Inoki *et al.*, 2005). In mammals, two genes exist for *Rheb- Rheb1* and *Rheb2(RhebL1)* (Patel *et al.,* 2003) which have a high degree of sequence homology at the protein level. In order to understand the function of this protein in mammalian systems, we sought to create a knockout mouse model for the *Rheb1* gene. Our accomplishments during the current funding period are:

(i) We have generated mice with *Rheb1* gene altered. However, further characterization led us to conclude that the mice we generated are not a complete gene replacement we initially envisioned. Instead, they appear to have the gene altered by the insertion of the neo cassette into a region adjacent to the exon2. The gene altered mice express a significantly decreased level of Rheb1.

(ii) We have generated antibody specific for mouse Rheb2. Our aim here was to have reagents valuable for characterizing cells derived from Rheb2 knockout mice once they are generated. The antibody has been affinity purified and characterized.

During the current funding period, our collaborator, Dr. Steve Young, relocated from the Gladstone Institute/UCSF to UCLA. This is a welcome event that gives tremendous boost to this project; we are now in the same location that should facilitate coordination between the Tamanoi lab and Young lab. However, because of the relocation, we suffered delay in starting our project.
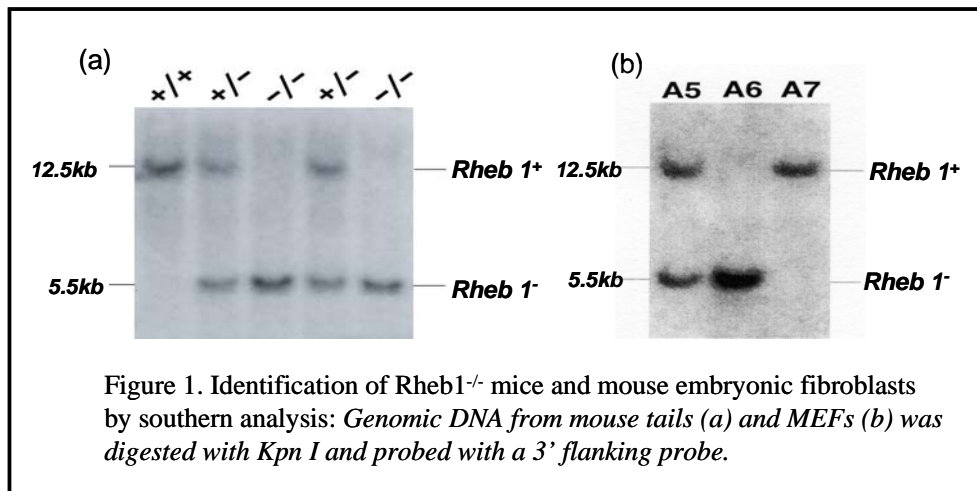

Detailed description of accomplishments

(a) Generation of Rheb knockout mice

We have generated Rheb1 gene altered mice

Previously, we designed a suitable knockout vector which was successfully used to generate *Rheb1* (-/-) homozygous embryonic stem (ES) cells as confirmed by southern blot using a 3' genomic probe. The sequence-replacement gene targeting vector was designed to replace exon 2 of the *Rheb1* gene with a neomycin phosphotransferase cassette. Targeted clones can be identified by Southern blot analysis with a 3' flanking probe. A 12.5-kb *Kpn* I band in a wild-type allele is converted to a 5.5-kb *Kpn* I band in the *Rheb1* knockout allele. Subsequently, these ES cells were used to generate *Rheb1*(-/-) mice. A knockout model for the *Rheb1* gene was successfully generated and presently we have both *Rheb1*(+/-) and (-/-) mice for further analyses.
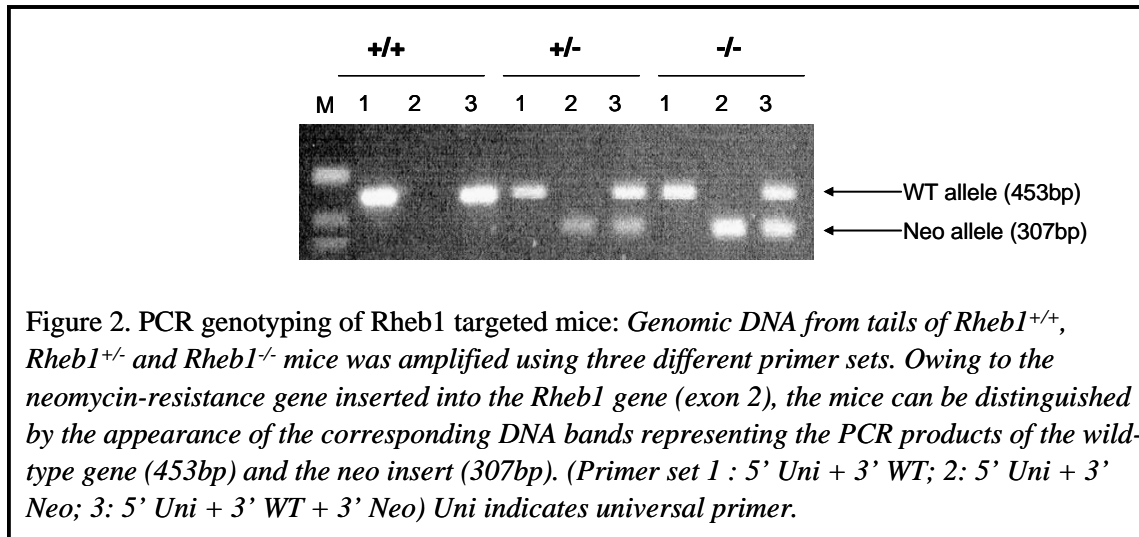
The generation of the mice was confirmed by carrying out a Southern analysis using genomic DNA from mouse tails in order to determine the *Rheb1* allele (Figure 1a). As observed, a 12.5-kb allele in the wild-type tail samples is converted into a 5.5-kb allele in the knockout tail DNA samples using a 3' probe. A similar observation was made using DNA isolated from mouse embryonic fibroblasts (E15.5) (Figure 1b).

Figure 1. Identification of Rheb1$^{-/-}$ mice and mouse embryonic fibroblasts by southern analysis: *Genomic DNA from mouse tails (a) and MEFs (b) was digested with Kpn I and probed with a 3' flanking probe.*

Rheb1 gene alteration was due to the insertion of neo cassette rather than gene replacement
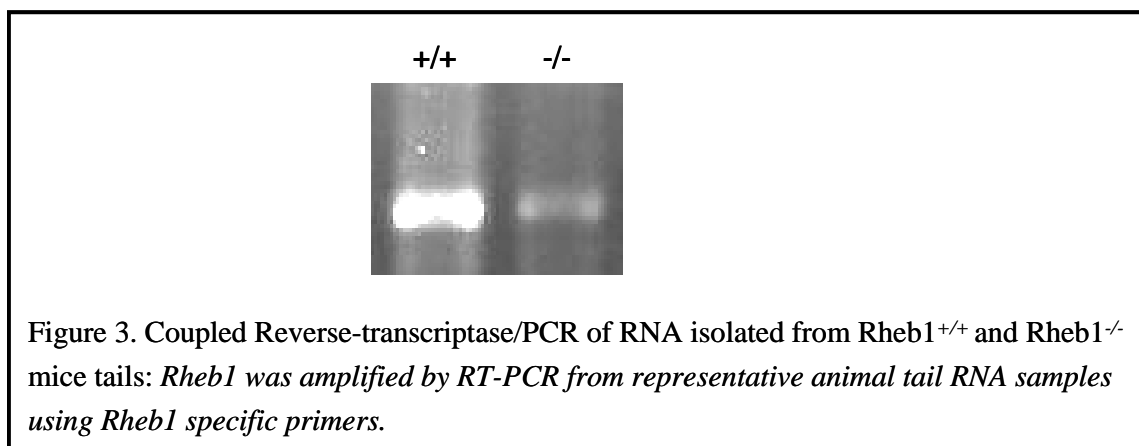
Further characterization led us to conclude that the *Rheb1* gene alteration we accomplished above represents insertion of the neo cassette rather than gene replacement. We realized this first when we genotyped tail samples. Our knockout strategy was to integrate the neomycin resistance gene into exon 2 of the *Rheb1* gene. Genotyping was carried out by PCR analysis using DNA from tail samples. The technique involved the use of primers specific to the wild-type as well as the neo allele to discriminate between the various *Rheb1* alleles. As observed in Figure 2, the *Rheb1*$^{+/+}$ (wild-type) mice exhibit a single band (453bp) while the *Rheb1*$^{+/-}$ (heterozygous) mice exhibit two bands (453bp for the wild-type allele and 307bp for the neo allele). To our surprise, two bands for the *Rheb1*$^{-/-}$ mice were also observed. A complete knockout should have yielded a single band at 307bp. This suggests that the exon2 was not removed in the mice we generated. This conclusion was further supported by PCR analysis to look for the presence of exon2. Tail DNA from the +/+ and +/- mice was analyzed for the presence of exon 2 (72bp) of the *Rheb1* gene which was the sequence targeted to be replaced by the neo cassette. PCR amplification was conducted using *Rheb1* exon 2 specific primers. The

expected 72bp fragment was observed in the +/+ samples, however this product was also amplified from the -/- samples (data not shown).
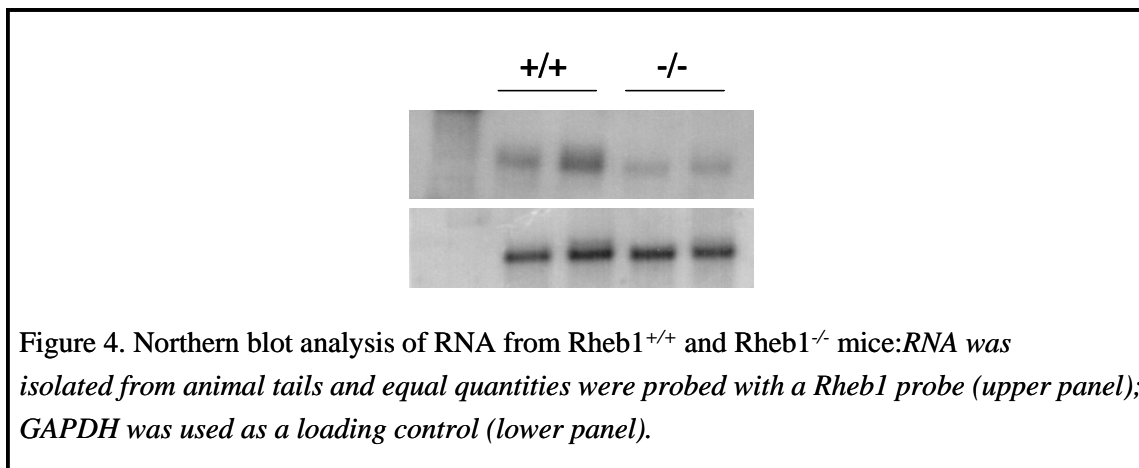

Figure 2. PCR genotyping of Rheb1 targeted mice: *Genomic DNA from tails of Rheb1$^{+/+}$, Rheb1$^{+/-}$ and Rheb1$^{-/-}$ mice was amplified using three different primer sets. Owing to the neomycin-resistance gene inserted into the Rheb1 gene (exon 2), the mice can be distinguished by the appearance of the corresponding DNA bands representing the PCR products of the wild-type gene (453bp) and the neo insert (307bp). (Primer set 1 : 5' Uni + 3' WT; 2: 5' Uni + 3' Neo; 3: 5' Uni + 3' WT + 3' Neo) Uni indicates universal primer.*

Rheb1 gene expression is significantly decreased in the mice we generated

The presence of exon 2 in the *Rheb1$^{-/-}$* mice led us to determine whether the *Rheb1* message is, in fact, being made in these mice or not. RNA was isolated from the +/+ and -/- animal tails and subjected to RT-PCR whereby the RNA was reverse transcribed to yield cDNA which was then amplified by PCR using *Rheb1* specific primers. As observed in Figure 3, a product was detected in both wild-type and -/- samples which suggested that *Rheb1* was being expressed in the -/- mice. In mammals, a second *Rheb* gene called *Rheb2* exists. To eliminate the possibility that the RT-PCR product observed in the -/- sample was not a non-specific cross-reactivity with the *Rheb2* gene, the DNA fragment was amplified and sequenced. Sequencing confirmed that the product did correspond to *Rheb1* and not *Rheb2*.

Figure 3. Coupled Reverse-transcriptase/PCR of RNA isolated from Rheb1$^{+/+}$ and Rheb1$^{-/-}$ mice tails: *Rheb1 was amplified by RT-PCR from representative animal tail RNA samples using Rheb1 specific primers.*

To determine the relative expression levels of the *Rheb1* gene in the +/+ and -/- animals, northern analysis was done. Equal quantities of total RNA isolated from +/+ and -/- animal tails were loaded and probed with a *Rheb1* specific probe (Figure 4). GAPDH was used as the loading control. As indicated in Figure 4, *Rheb1* expression was detectable in both +/+ and -/- samples, however, the level was much reduced in the -/- animals. Thus, it appears that we have generated a hypomorphic allele of *Rheb1* rather than a complete knockout model. The actual protein levels of the Rheb1 protein in the animals could not be analyzed due to the absence of specific antibodies toward mouse Rheb1. The *Rheb1*$^{-/-}$ mice we generated did not exhibit growth defects or phenotypic changes and two reasons could be attributed for these observations- one, a small amount of Rheb1 may be sufficient to fulfill its functional contribution, hence a hypomorphic allele would not display any phenotypes and second, the *Rheb2* gene may have a redundant role and could be compensating for the reduction in Rheb1 expression. If Rheb2 is, in fact, capable of rescuing loss of Rheb1, it may be crucial to develop a double knockout *Rheb* model or a conditional allele for both genes.


Figure 4. Northern blot analysis of RNA from Rheb1$^{+/+}$ and Rheb1$^{-/-}$ mice:*RNA was isolated from animal tails and equal quantities were probed with a Rheb1 probe (upper panel); GAPDH was used as a loading control (lower panel).*

(b) Generation of an antibody specific toward mouse Rheb2

Mouse Rheb1 and Rheb2 proteins are highly conserved with a 51% identity at the amino acid level. To date no study has been conducted to analyze the endogenous Rheb2 protein levels in mammalian cell lines or tissues. A number of antibodies are available against the human Rheb1 protein (Cell Signaling, Santa Cruz) but no antibody exists against the Rheb2 protein. We

sought to develop an antibody against mouse Rheb2 with an intention to detect the endogenous protein and utilize it specifically for Rheb2 with minimal cross-reactivity toward Rheb1.

We have generated antibody against Rheb2

In order to raise an antibody which is specific for mouse Rheb2 exclusively, the protein sequences were examined to delineate regions of highest variability. The region with least homology was found to be at the C-terminus of the protein. A 14-residue C-terminal peptide spanning from amino acid 167 to 180 was selected as the epitope to generate the antibody. Within this peptide stretch, there is no amino acid identity between the two proteins and hence was deemed most suitable to serve as the antigen (Figure 5a). The antibody was synthesized by Washington Biotechnology (Maryland) in two New Zealand rabbits and subsequently affinity purified.

Figure 5 (a) Alignment of mouse Rheb1 and Rheb2 protein sequences. (b) C-terminal peptide sequences of mouse and human Rheb2
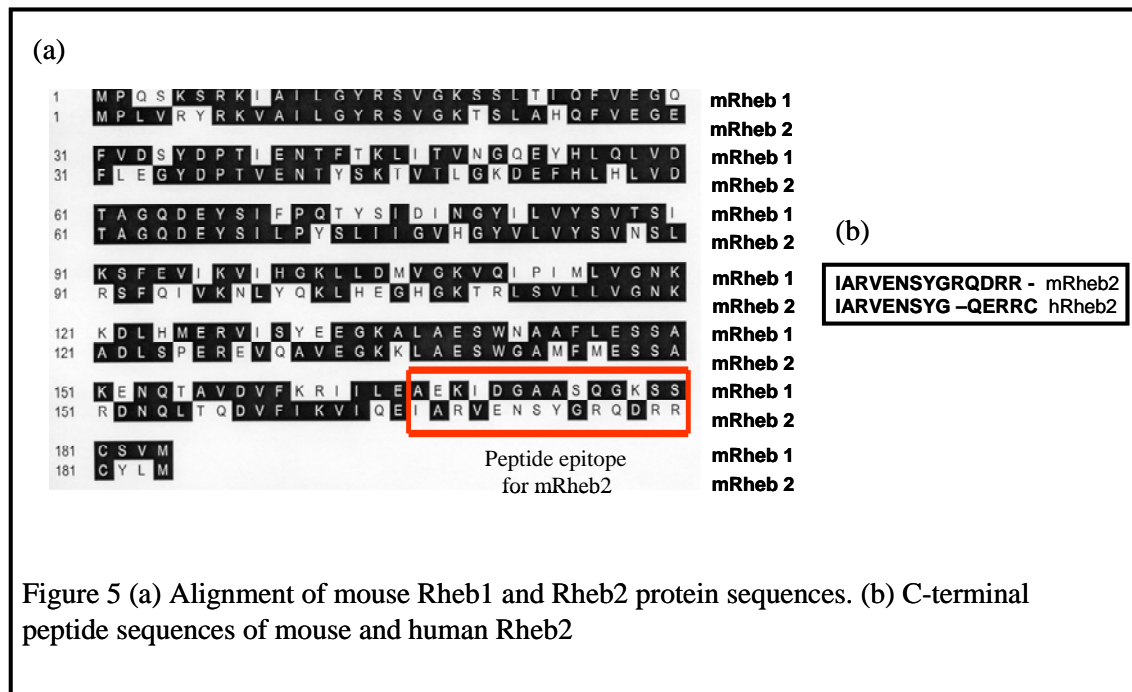
Our antibody specifically recognizes mouse Rheb2

The affinity purified antibody was tested against a variety of purified G-proteins to determine its specificity and was found to be highly specific for mouse Rheb2 with no cross-reactivity toward other proteins (Figure 6a). The antibody was then examined for its reactivity with Rheb proteins from various species. Purified recombinant human, mouse, *Drosophila* and *S. pombe* proteins were tested as potential antigens as shown in Figure 6b (lower panel). The antibody reacted with mammalian Rheb2 (mouse protein) specifically and did not recognize

Rheb from other species. Surprisingly, the antibody was found not to cross-react with recombinant human His-Rheb2 as well. Comparison of the amino acid sequence within the antigenic peptide between the human and mouse Rheb2 proteins revealed an 85% identity (Figure 5b) although gaps were found between the regions of homology. A similar study using an antibody (Cell Signaling) toward the human Rheb1 protein showed that this antibody was reactive toward both mouse Rheb1 and Rheb2, although the signal was found to be stronger for mRheb1 (Figure 6b, upper panel). Thus, the antibody we generated is highly specific for Rheb2 and selective for mouse Rheb2 alone with little cross-reactivity against either Rheb1 or human Rheb2.

Figure 6 (a) Reactivity of antibody toward mouse Rheb2 against several G-proteins. (b) Specificity of α-human Rheb1 *(upper panel)* and α-mRheb2 *(lower panel)* against Rheb proteins from various species.
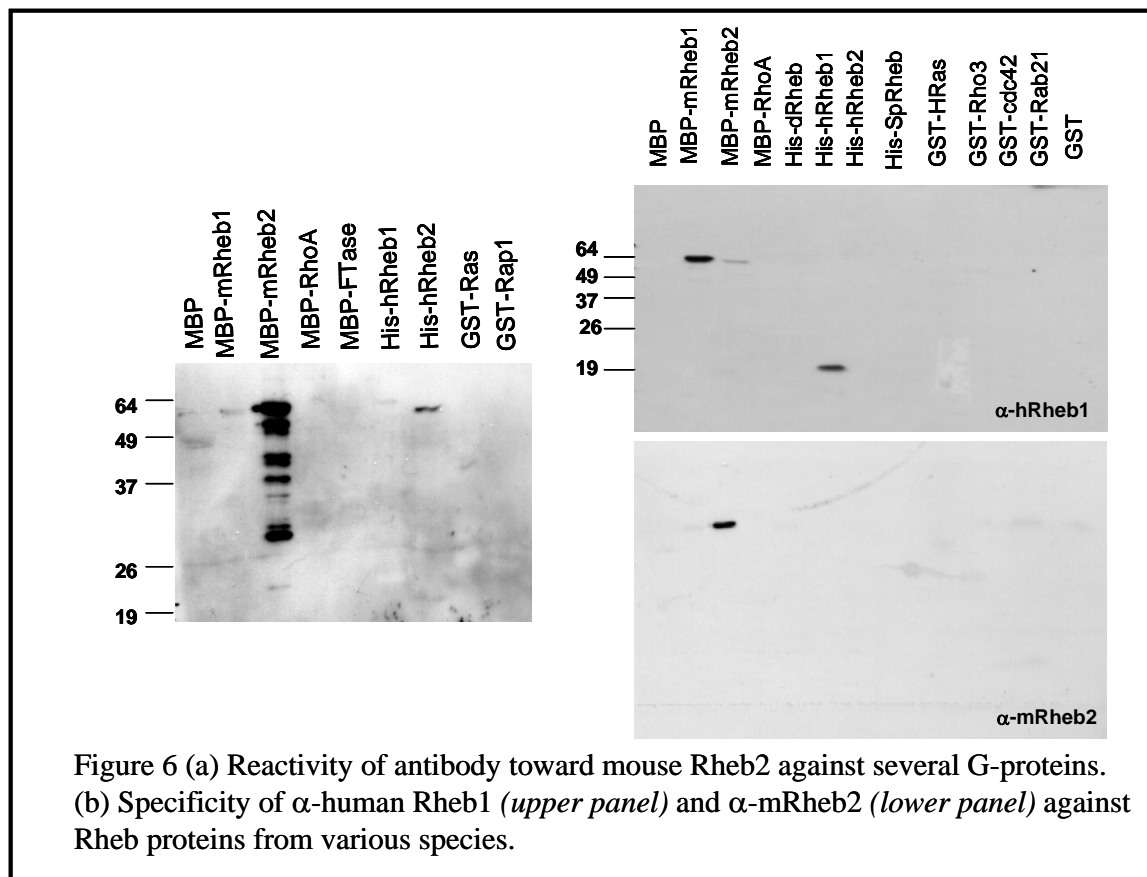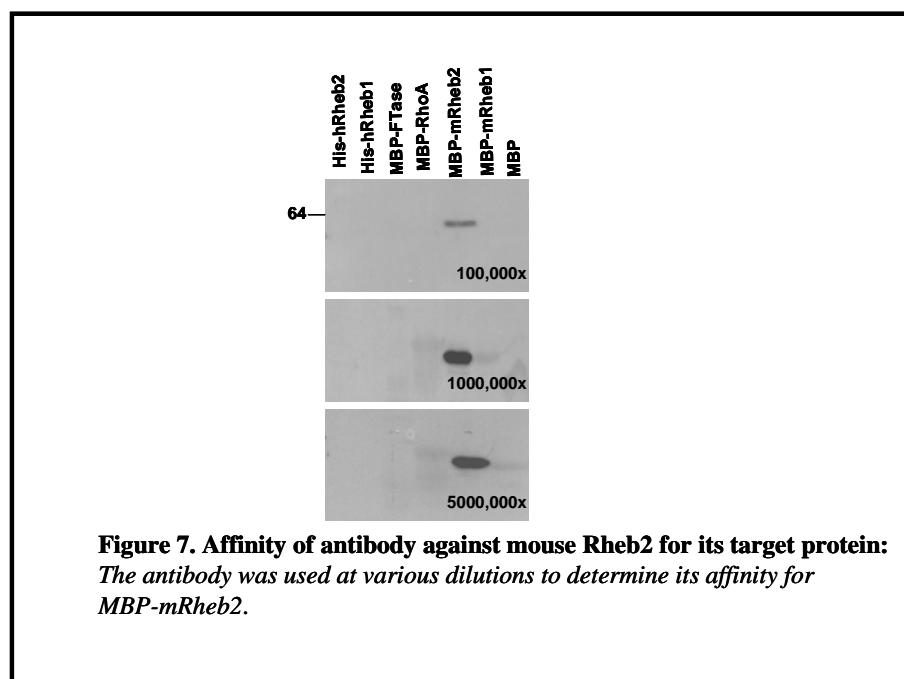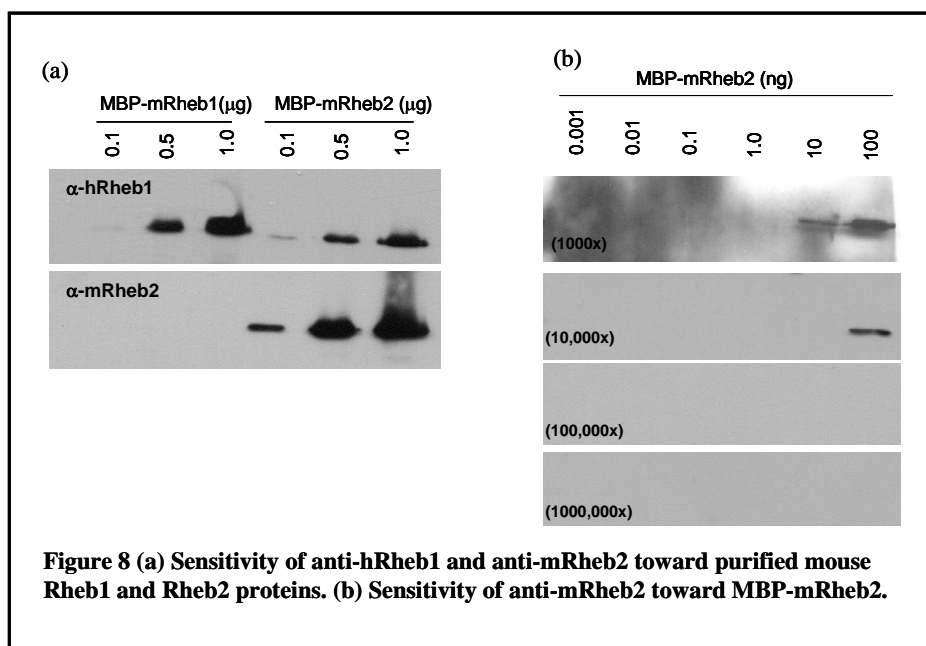
Figure 7 shows that the antibody has high affinity for its target mRheb2 protein. The antibody was tested at dilutions ranging from 100,000 to 5000,000 fold and was found to react with Rheb2 even at very high dilutions.
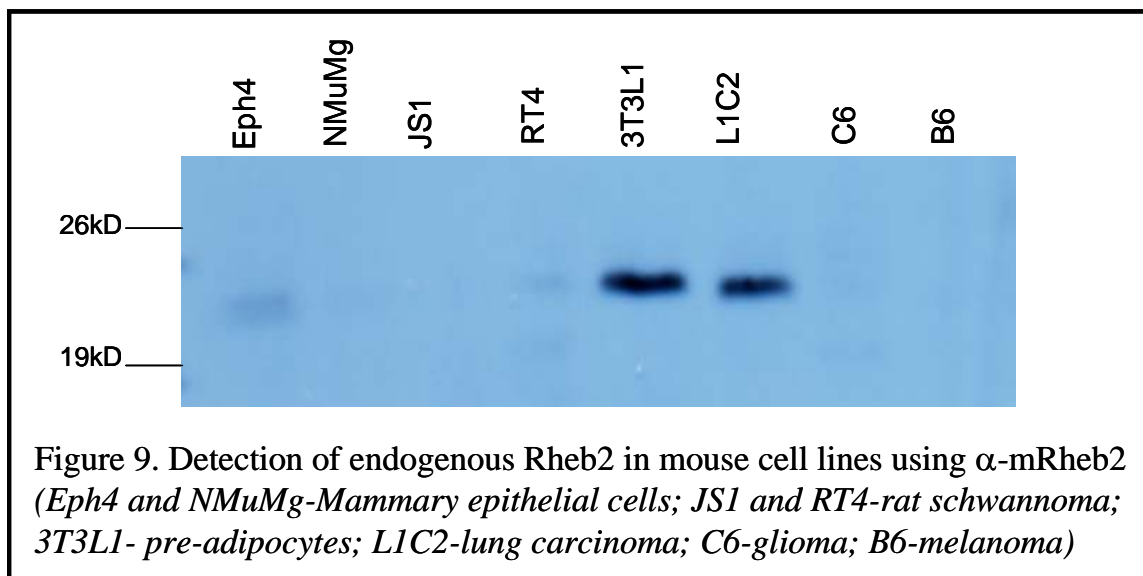
Figure 7. Affinity of antibody against mouse Rheb2 for its target protein: *The antibody was used at various dilutions to determine its affinity for MBP-mRheb2.*

The sensitivities of both α-human Rheb1 and α-mRheb2 were analyzed using varying amounts of purified recombinant MBP-mRheb1 and 2 proteins (0.1 to 1 μg). As observed in Figure 8a, the anti-mRheb2 was capable of detecting 100 ng of its target protein unlike the human Rheb1 antibody which was able to detect the target protein at 500ng and above. To define the sensitivity thresholds of anti-Rheb2 further, the concentration of the antigen protein was lowered to picogram levels and the antibody was tested at various dilutions. As shown in Figure 6b, the antibody could detect as low as 10 ng of mRheb2 when used at a dilution of a 1000 fold. The high affinity of the antibody for Rheb2 suggests that it may serve as a useful tool for detecting endogenous Rheb2 in cell lines/tissues with minimum background reactivity with Rheb1.

Figure 8 (a) Sensitivity of anti-hRheb1 and anti-mRheb2 toward purified mouse Rheb1 and Rheb2 proteins. (b) Sensitivity of anti-mRheb2 toward MBP-mRheb2.

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Detection of endogenous Rheb2 protein

Various mouse cell lines were then analyzed to determine if endogenous Rheb2 could be detected. As shown in Figure 9, endogenous Rheb2 could be detected in three cells lines- 3T3-L1 pre-adipocytes, L1C2 lung carcinoma cells and PC12 (pheochromocytoma) cells. This is the first study reporting the detection of endogenous mouse Rheb2 in cell lines and suggests that the antibody is, in fact, capable of reacting with cellular Rheb2. This finding is interesting, as it has been reported that Rheb2 has a limited expression pattern. Presently, the antibody is being utilized fur studying mouse tissues and several other cell lines.

Figure 9. Detection of endogenous Rheb2 in mouse cell lines using α-mRheb2 *(Eph4 and NMuMg-Mammary epithelial cells; JS1 and RT4-rat schwannoma; 3T3L1- pre-adipocytes; L1C2-lung carcinoma; C6-glioma; B6-melanoma)*

Currently ongoing projects and plans for the next funding period

Although the *Rheb1* gene altered mice we generated provide a valuable system to examine consequences of decreasing *Rheb1* expression, it is necessary to generate a complete knockout of *Rheb1*. Towards this goal, we have initiated another attempt to generate knockout mice. The *Rheb1* knockout vector has been transfected into ES cells and the ES cells are currently being examined to identify knockout clones. This time, we are carrying out a variety of methods to confirm gene replacement before injecting the targeted ES clones into mice. Southern analyses are currently being carried out using probes against the regions 5' and 3' to exon2. We expect to obtain ES cells with complete gene replacement which can then be introduced into mice.

In addition, we have been generating *Rheb2* knockout mice. The knockout vector for *Rheb2* has been constructed. After detailed characterization of the vector, it was transfected into ES cells. Currently, the ES cells are being characterized to identify *Rheb2* knockout clones. We will carry out detailed analysis of the ES cells to confirm that the *Rheb2* gene has been replaced. The ES cells will then be introduced into mice.

While Rheb1 is ubiquitously expressed (Gromov *et al.*, 1995), Rheb2 (human) has been reported to exhibit a limited expression profile (Saito *et al.,* 2005). Therefore, it is important to learn more about Rheb2 while we are generating *Rheb2* knockout mice. Knowledge of tissue expression of Rheb2 may provide information that may be relevant for understanding phenotypes of *Rheb2* knockout mice. The antibody we generated against mouse Rheb2 is being used to examine tissue expression patterns of Rheb2. In addition, we plan to examine the expression of Rheb2 during development of mouse embryos.

The availability of an antibody against mouse Rheb2 enabled us to detect endogenous Rheb2 protein. It will be interesting to examine the posttranslational modifications of this protein if they exist. To investigate this point, we are currently purifying endogenous Rheb2 protein using immunoaffinity purification. The purified materials will be characterized.

Key Research Accomplishments

We have accomplished the following:

1. We have successfully generated mice with an altered Rheb1 gene expression which are presently being analyzed further.

2. We have succeeded in generating an antibody specific toward mouse Rheb2 which shows minimal cross-reactivity for Rheb1 and could serve as a useful tool for the study of endogenous Rheb2.

Reportable outcomes

1.   Mice with an altered Rheb1 gene expression have been generated.

2.   An antibody highly specific for mouse Rheb2 has been synthesized.

Conclusions

During the current funding period, we have accomplished:

1. Generation of mice with reduced expression of Rheb1.
2. Production of an antibody specific toward mouse Rheb2.

In addition to further investigating Rheb function using the above reagents, we will generate other Rheb1 mice as well as Rheb2 knockout mice. Studies should provide important insights into the role of Rheb GTPase in Tuberous Sclerosis.

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